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Expression of a Gene Specifically Expressed in *Dictyostelium* Prestalk Cells

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In the *Dictyostelium* slug there are two types of prestalk cells, pstA cells and pstO cells, that differ in their ability to utilize the distal and proximal parts of the promoter of *ecmA*, a gene that is specifically expressed in prestalk cells. When Rm, a dominant inhibitory form of the regulatory subunit of cAMP-dependent protein kinase (PKA), is expressed under the control of the complete promoter of the *ecmA* gene (in a construct termed ecmAO:Rm) development proceeds to the slug stage. Although able to form small but outwardly normal slugs, ecmAO:Rm cells are defective in prestalk cell differentiation. In ecmAO:Rm cells, the induction of pstA- and pstO-specific gene expression by the stalk cell inducer DIF is greatly inhibited. Paradoxically, a very large fraction of the cells in an ecmAO:Rm slug show evidence of once having expressed the ecmA and ecmO prestalk markers. However, we present evidence that this is due to abortive prestalk cell differentiation that terminates when sufficient Rm protein has accumulated to block PKA activity. This results in regulative transdifferentiation of prespore cells to form prestalk cells. During their transitory period as prestalk cells the ecmAO:Rm cells coexpress both the ecmA and ecmO markers, indicating a possible link between PKA activity and divergence of the two prestalk cell subtypes. Finally, we show that the level of the DNA binding activity believed to lie at the end of the DIF signal transduction pathway is reduced in ecmAO:Rm slugs.

INTRODUCTION

The *Dictyostelium* slug is an intermediate in development containing cells that are part way along either the stalk or the spore differentiation pathways, but which have not become irreversibly committed to their presumptive fates (Raper, 1940). This flexibility is presumably required because, as the slug migrates, there is a gradual loss of prestalk cells (Sternfeld, 1992) and there must be the potential for the transdifferentiation of prespore into prestalk cells if the correct proportion of cell types is to be maintained. Regulative behavior implies the existence of diffusible signals that pass between the cells to determine their state of differentiation and it seems most likely that the molecules that induce initial cellular differentiation also form part of the regulation mechanism. Two such molecules have been identified, cAMP and a chlorinated hexaphenone called DIF.

The prespore cells are located in the rear four-fifths of the slug and their differentiation is induced and maintained by extracellular cAMP signaling (Kay *et al.*, 1978; Barklis and Lodish, 1983; Mehdy *et al.*, 1983; Oyama *et al.*, 1982; Schaap and van Driel, 1985). These signals are perceived by cell surface receptor molecules which are coupled to heterotrimeric G proteins that activate a number of intracellular effectors, including adenylate cyclase (reviewed in Firtel *et al.*, 1989; Hereld and Devreotes, 1992). Changes in intracellular cAMP are perceived by the cAMP-dependent protein kinases (PKA) holoenzyme which, in *Dictyostelium*, contains a single regulatory (R) and a single catalytic (C) subunit (De Gunzburg and Veron, 1982; Mutzel *et al.*, 1987). Rm is a mutant form of the R subunit that contains a point mutation in each of the two cAMP binding sites and which acts as a dominant inhibitor of the C subunit (Harwood *et al.*, 1992a). When Rm is placed under the control of the promoter of *pspA*, a gene encoding a cell surface protein specific to prespore cells, the expression of *pspA* and a number of other genes specifically expressed in prespore cells is repressed (Hopper and Williams, 1994; Hopper *et al.*, 1995). This occurs by a combination of transcriptional and posttranscriptional regulation. Thus, a certain level of

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PKA activity is required for correct prespore cell differentiation but further activation of PKA, presumably via a rise in intracellular cAMP, is both necessary and sufficient to induce the maturation of prespore cells into spores (Kay, 1989; Anjard *et al.*, 1992; Hopper *et al.*, 1993, 1995; Mann and Firtel, 1993; Mann *et al.*, 1994; Richardson *et al.*, 1994).

Prestalk cells are located in the front one-fifth of the slug and their differentiation is induced by DIF, a secondary metabolite that accumulates during multicellular development (Brookman *et al.*, 1982; Kay and Jermyn, 1983; Morris *et al.*, 1987). Part of the evidence for DIF's role in prestalk induction comes from studying the *ecmA* gene, which encodes a protein of the extracellular matrix that surrounds the slug (McRobbie *et al.*, 1988). The *ecmA* gene is expressed only in prestalk cells and its transcription is rapidly induced by DIF (Williams *et al.*, 1987).

The intracellular signaling pathway that mediates DIF induction of prestalk cell differentiation is largely uncharacterized. However, there is a developmentally regulated DIF binding protein that is partly cytosolic and partly nuclear (Insall and Kay, 1990) and a wholly nuclear activity that binds to an element within the ecmA promoter that is essential for prestalk-specific, DIF-inducible gene expression (Kawata *et al.*, 1996).

Activation of PKA is both necessary and sufficient to induce stalk cell differentiation. Overexpression of the C subunit of PKA, or inactivation of the R subunit, leads to ectopic stalk cell differentiation *in vivo* and to stalk cell differentiation in cells incubated in monolayer (Simon *et al.*, 1992; Anjard *et al.*, 1992; Hopper *et al.*, 1993; Mann *et al.*, 1993). Treatment of cells with 8Br-cAMP, a membrane-permeant analog of cAMP, also induces stalk cell differentiation in cells incubated in monolayer (Maeda, 1988; Inouye and Gross, 1993; Kubohara *et al.*, 1993).

Conversely, inhibiting PKA prevents stalk cell differentiation. When Rm is expressed from the entire ecmA promoter (in the construct we term ecmAO:Rm), development arrests at the slug stage. However, strains containing a fusion gene where the ecmA promoter directs expression of Rc, a mutant form of the R subunit that cannot interact with the C subunit, develop perfectly normally (Harwood *et al.*, 1992b). The ecmAO:Rm strains behave as ''sluggers,'' mutants that remain as migratory slugs for extended periods of time (Sussman *et al.*, 1978; Newell and Ross, 1982). While the ecmAO:Rm slugs sometimes rear on end and attempt to culminate, the cells are incapable of terminal differentiation. Also, when ecmAO:Rm cells are incubated as a monolayer, DIF will not induce terminal stalk cell differentiation (Harwood *et al.*, 1992b).

It is important to determine at which stage in the stalk cell pathway of differentiation PKA exerts its effects. Is it only affecting the differentiation of prestalk into stalk cells or is it also affecting the formation of prestalk cells? Both the behavior of the ecmAO:Rm slugs and their pattern of gene expression suggest that PKA might be necessary for normal prestalk cell differentiation. The front half of the prestalk zone, the tip region, seems to be defective in at least two ways. EcmAO:Rm slugs are about half to one-third the size of control slugs (Bonner and Williams, 1994) and extracellular cAMP signals from the tip are believed to determine slug size (evidence reviewed by Wang and Schaap, 1985). The tip also controls phototaxis (Francis, 1964) and ecmAO:Rm slugs are severely defective in this aspect of their behavior (Bonner and Williams, 1994).

Northern transfer shows the *ecmA* gene to be reduced in its expression by a factor of about fivefold in ecmAO:Rm slugs (Harwood *et al.*, 1992b) but is, in this case, an inadequate method of assaying cellular differentiation because it does not give information about the level of expression in individual cells. Are there fewer prestalk cells or does each cell express the ecmA gene more weakly? Also, there are now known to be two different kinds of prestalk cells, which differ in their positions within the slug: the pstA cells occupying the slug tip with the pstO cells lying between the pstA cells and the prespore region (Jermyn *et al.*, 1989). It is important to know how their differentiation is affected when PKA is inhibited.

The promoter of the *ecmA* gene contains different regions that are responsible for directing expression in the pstA and pstO cell populations and these promoter regions, fused to the *lacZ* gene, provide markers of their differentiation (Early *et al.*, 1993, 1995). Here we investigate the role of PKA in the differentiation of pstA and pstO cells. We show that PKA activity is required for differentiation along both pathways and present evidence that it may also be involved in the specification of pstA and pstO cells as discrete subtypes. Finally, we show, by a gel retardation assay, that the level of the DNA binding activity, which is believed to regulate prestalk-specific gene expression (Kawata *et al.*, 1996), is reduced in ecmAO:Rm slugs. This suggests that this protein lies within a PKA-regulated signal transduction pathway.

MATERIALS AND METHODS

Construction of plasmids. The construction of ecmAO:Rm (originally called ecmA:Rm) is described in Harwood *et al.* (1992a,b). The structure of ecmO:lacZ is described in Early *et al.* (1993) and that of ecmA:lacZ in Early *et al.* (1995). The ecmO:GFP construct was made by transferring the *Xba*I–*Bgl*II fragment from ecmO:lacZ encompassing the promoter region into the *Xba*I–*Bgl*II sites of the multilinker of pDdgfp (Gerisch *et al.*, 1995). To construct pspA:GFP the *Bgl*II–*Xho*I fragment from pDdgfp, spanning the *gfp* gene, was used to replace the *lacZ* gene in pspA:lacZ (Detterbeck *et al.*, 1994).

Cell culture, transformation, and development. Dictyostelium discoideum cells of the AX-2 strain were grown and transformed as described previously (Watts and Ashworth, 1970; Early and Williams, 1987), except that in final selection G418 was present at 200 μg/ml. Cotransformant clones, containing ecmAO:Rm and either ecmO:lacZ or ecmA:lcaZ, were selected by plating amoebae in association with *Klebsiella aerogenes* and were screened visually for the ecmAO:Rm phenotype (a failure to culminate). The clones with a strong phenotype were further selected, for a high level of gene expression, by screening for β-galactosidase activity by an *in situ* detection method (Buhl *et al.*, 1993). Cells of the strain HMX44 (Morrison and Harwood, 1992), an axenic derivative of HM44 (Kopachik *et al.*, 1983), were transformed as for AX-2 cells except that

in the latter stages of the selection G418 was present at 20 μg/ml (HMX44 is more highly sensitive to G418 than AX-2). The analysis of gene expression of the HMX44 transformants was performed on pooled populations, to correct for copy number effects of the reporter constructs.

Development was performed by washing exponentially growing cells in KK2 (16.5 m*M* KH_2PO_4, 3.8 m*M* K_2HPO_4, pH 6.2) and plating them at a density of 5×10^7/ml on white nitrocellulose filters supported on 2% water agar plates (Difco, ''Bacto Agar''). Plates were incubated at 22°C in a humid chamber. For analyzing the fraction of cells expressing prestalk or prespore markers at the slug stage, or for performing grafting experiments, cells were plated at a density of 10^8/ml in thin streaks on 2% water agar plates. These were incubated next to a low-level, unidirectional light source to allow the slugs to migrate away from the origin. After 2 to 4 hr of migration grafting was performed using a hair loop.

Analysis of gene expression. Filters bearing cells at the required stage of development were transferred to 1% glutaraldehyde in Z buffer (60 m*M* Na_2HPO_4, 40 m*M* NaH_2PO_4, 10 m*M* KCl, 1 m*M* $MgSO_4$, 2 m*M* $MgCl_2$) for 10 min and washed twice in Z buffer, without fixative but containing 0.2% Tween 20, and once in Z buffer with no additive (Dingermann *et al.*, 1989). They were then incubated in Z buffer containing 5 m*M* $K_3(Fe(CN)_6)$, 5 m*M* $K(Fe(CN)_6)$, and 1 m*M* X-gal at 22°C until the appropriate degree of staining was obtained. The reactions were stopped by replacing the staining solution with Z buffer. At the end of the experiment the developing structures were washed off the filters using gelvatol and placed on a glass slide for photography.

Slugs were dissociated into single cells by trituration through progressively smaller-bore syringe needles. Prestalk-specific gene expression was assayed by fixing the cells for 10 min in an Eppendorf tube with Z buffer containing 0.05% glutaraldehyde and staining as above. Prespore-specific gene expression was assayed by staining with an antibody that recognizes vesicles that are present only in prespore cells and that contain spore coat components (Takeuchi and Sakai, 1971). Dissociated cells were placed onto glass slides that had been coated with poly-L-lysine and then fixed in methanol for 10 min. They were stained with a polyclonal rabbit antiserum directed against *D. mucoroides* spores (Takeuchi and Sakai, 1971; a kind gift of Dr. R. Kay). TRITC-labeled, goat anti-rabbit antibody was used as a secondary detection reagent. After washing (3× 5 min) with PBS (0.14 *M* NaCl, 3 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4, pH 7.2), the samples were mounted in gelvatol and visualized using a confocal microscope (Bio-Rad, Model MRC1000).

Double labeling of dissociated cells was performed by fixing cells in glutaraldehyde and staining with X-gal as above. After 24 hr of staining, the cells were secondarily fixed by immersion in methanol at room temperature for 10 min. They were then incubated for 24 hr at 4°C in a primary antibody solution containing MUD-1 (1 μg/ml), a mouse monoclonal antibody directed against the PSPA protein (Krefft *et al.*, 1983). Following several washes in PBS, the slides were incubated with a secondary antibody solution containing FITC-conjugated anti-mouse IgG antibody for an additional 24 hr at 4°C. After washing with PBS (3× 5 min), the samples were mounted in gelvatol. Images of the cells were recorded using a video camera mounted on an orthodox light microscope. Fluorescence images of the same fields were then recorded using the confocal microscope.

Induction of marker gene expression by DIF. Subconfluent HMX44 cells, transformed with either the ecmA:lacZ marker or the ecmO:lacZ marker, were washed twice with KK2 and plated in submerged monolayer culture in stalk salts buffer (Kopachik *et al.*, 1985; 10 m*M* 4-morpholineethane sulfonic acid (Mes), 2 m*M* NaCl, 10 m*M* KCl, 1 m*M* $CaCl_2$, 5 m*M* cAMP, 200 μg/ml streptomycin sulfate, and 15 μg/ml tetracycline, pH 6.2). Incubation at a density of 2×10^5/ml was in the presence of 5 m*M* cAMP for the first 8 hr. The cells were then washed three times with stalk salts buffer before addition of buffer containing 5 m*M* cAMP or additionally supplemented with DIF at a concentration of 50 n*M* (a gift of R. Kay). After a further 18 hr, the cells were fixed *in situ* and stained overnight at 37°C using X-gal as described above.

Isolation of nuclear extract and gel retardation assay. The extract was prepared exactly as described previously (Kawata *et al.*, 1996), from axenically growing cells which were allowed to develop on water agar to a time immediately after slug formation. Briefly, a crude nuclear extract was prepared exactly as described by Insall and Kay (1990) except that the concentration of NP-40 was 1%. A 20–60% ammonium sulfate fraction was prepared and the pellet was dissolved in dialysis buffer (10% glycerol, 50 m*M* potassium phosphate, pH 7.5, 2 m*M* $MgCl_2$, 400 m*M* KCl, 200 μ*M* TLCK, 500 μ*M* PMSF, 1 m*M* DTT) and dialyzed against the same buffer. For the gel retardation assay, 2–4 μg of fractionated nuclear extract was used. Binding reactions contained 5 μl of the extract diluted with dialysis buffer, 2 μl of binding buffer (20 m*M* Hepes–KOH, pH 7.9, 100 m*M* NaCl, 2 m*M* $MgCl_2$, 1 m*M* EDTA, 40% glycerol, 2 m*M* DTT, 1 m*M* PMSF, and 0.02% NP-40), 0.5 μg of poly(dA − dT)·poly(dA − dT) (Pharmacia), and 2 ng of probe in a 10-μl total volume. Prebinding of any competitors was performed in an ice bath in the absence of the probe for 60 min and then the probe was added and the tube incubated at room temperature for 30 min. The mixture was then electrophoresed on a 5% native polyacrylamide gel.

The probe was made by annealing complementary oligonucleotides derived from nucleotides −1165 to −1218 of the ecmA promoter (Early *et al.*, 1993). These were both made with *Bgl*II cohesive ends and were labeled with [α-^{32}P]dATP (6000 Ci/mmol, Amersham) using the Klenow fragment of DNA polymerase I. Unincorporated nucleotides were removed by two sequential passages through a ''Push Column'' (Stratagene).

RESULTS

Analysis of cells in monolayers suggests that both pstA and pstO cell differentiation are repressed when PKA is inactive. Normal development is complicated by the cell–cell signaling that is occurring and one very valuable approach to minimize this complication is to study the differentiation of cells incubated as a monolayer (Bonner, 1970; Town *et al.*, 1976). The *Dictyostelium* strain HMX44 is defective in the production of DIF but remains responsive to it, so that cellular differentiation can be made dependent upon the addition of DIF (Kopachik *et al.*, 1983; Morrison and Harwood, 1992). HMX44 cells were cotransformed with ecmAO:Rm and with ecmA:lacZ, a construct that directs expression only in pstA cells, or with ecmO:lacZ, a construct that directs expression only in pstO cells (Early *et al.*, 1993, 1995). The cells were exposed to cAMP, to render them competent to respond to DIF, and then incubated with DIF for 18 hr, fixed, and stained with X-gal.

During incubation the cells aggregate into clumps and so in the control cells, i.e., those that do not contain ecmAO:Rm, it is not possible to count the number of stained

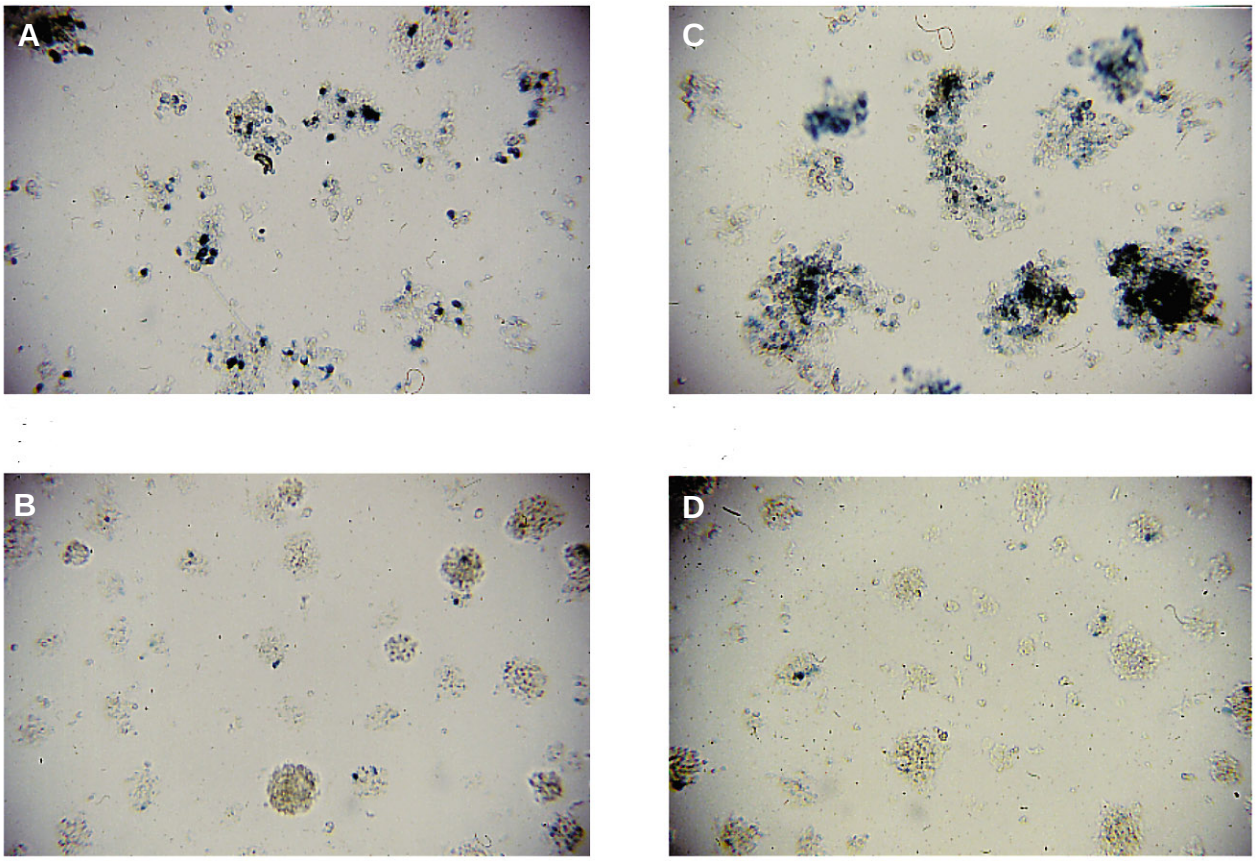
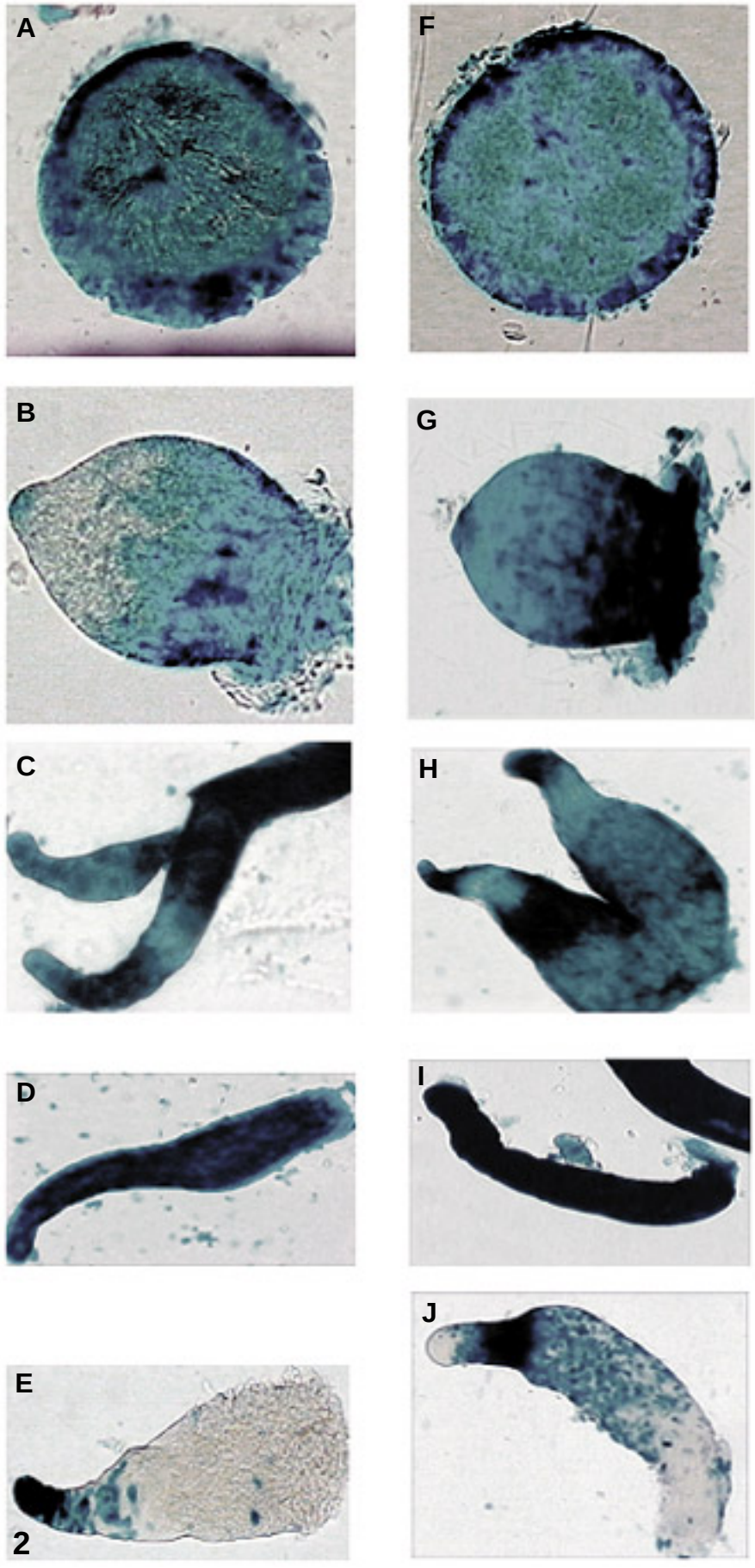


FIG. 1. Analysis of gene expression in cells induced with DIF in a monolayer. HMX44 cells were incubated in a tissue culture dish in a buffer containing cAMP for 8 hr and then incubated for a further 18 hr in the presence of DIF (see Materials and Methods). They were then fixed *in situ* and stained for β-galactosidase activity. (A) Cells transformed with ecmA:lacZ, (B) cells cotransformed with ecmA:lacZ and ecmAO:Rm, (C) cells transformed with ecmO:lacZ, (D) cells cotransformed with ecmO:lacZ and ecmAO:Rm. Because it is the most reliable way of comparing levels of gene expression between different transformants, we used pooled populations rather than clones and the cells expressing at a low level in B and D are presumably those where copy number is high enough to give a detectable signal. The average *Dictyostelium* cell (most clearly seen here as one of the blue objects in the DIF-treated samples) is approximately 10 μm in diameter. The data were collected with a zoom lens on a stereo microscope and we cannot therefore calculate a scale bar.

cells. However, it is clear that a very large fraction of them express the ecmA:lacZ (Fig. 1A) or ecmO:lacZ constructs (Fig. 1C), while in cells transformed with ecmAO:Rm there are very few cells within the clumps that detectably express either marker (Figs. 1B and 1D).

The results of Harwood *et al.* (1992b) show that PKA is required for optimal ecmA gene expression and these monolayer induction results imply an almost absolute requirement for PKA activity if a cell is to become a prestalk cell. How then is it possible for a slug to be formed from ecmAO:Rm cells? We believe that the answer to this apparent paradox lies in the fact that expression of Rm is under control of a prestalk-specific promoter, so that there is a window of opportunity, a time immediately after it differentiates as a prestalk cell when a cell is expressing only a small amount of the dominant inhibitor and so can display some of the properties of a prestalk cell. After this time, continued accumulation of the inhibitor causes it to lose these properties. A number of pieces of evidence support this conclusion; the first derives from studying the normal development of ecmAO:Rm transformant cells containing either the ecmA:lacZ or the ecmO:lacZ construct.

FIG. 2. Analysis of the development of ecmAO:Rm cells. AX-2 cells transformed with either ecmA:lacZ (A–E) or ecmO:lacZ (F–J) constructs were subjected to development and fixed and stained for β-galactosidase activity. The cells in A–D and F–I were cotransformants containing ecmAO:Rm and the ecmA:lacZ marker (A–D) or ecmO:lacZ marker (F–I) while the cells in E and J were transformed only with the ecmA:lacZ (E) or ecmO:lacZ (J) marker.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For ecmAO:Rm cells tip formation is delayed and ecmA- and ecmO-expressing cells become positioned ectopically. During slug formation cells aggregate together to form a mound-shaped structure called the tight aggregate. A nipple-shaped tip, composed of prestalk cells, appears at the top of the mound and this elongates so that eventually the mound is transformed into a cylindrical structure called the first finger. Under environmental conditions inappropriate for immediate culmination the first finger topples onto its side and moves away as a migratory slug.

Up to the loose aggregate stage ecmAO:Rm transformants appear normal but then development slows dramatically. While control cells go on to form a slug after a further 5 to 6 hr, the ecmAO:Rm aggregates take at least 12 hr to form a slug. Also, they display aberrant gene expression for both the pstA- and the pstO-specific markers. First, the level of expression relative to wild-type controls is low. Many ecmAO:Rm transformant clones had to be screened to find those with a usable level of expression and, even with such clones, it was necessary to stain longer than for wild-type aggregates.

The ecmAO:Rm aggregates are also highly aberrant in the localization of pstA and pstO cells, in that the two markers that distinguish them in wild-type cells, ecmA:lacZ and ecmO:lacZ, are coexpressed. The initial site of pstO cell differentiation is unknown but pstA cells appear to differentiate at the periphery of the aggregate and then rapidly move to the apex, where they come to form the tip (Early *et al.*, 1995). In ecmAO:Rm cotransformants containing either the pstA-specific or the pstO-specific lacZ marker there is enrichment of staining in a peripheral ring at the tight aggregate stage (Figs. 2A and 2F). Outwardly identical ecmA:lacZ and ecmO:lacZ staining patterns are also observed at all subsequent stages of development and this apparent coexpression is confirmed below by the analysis of individual cells. Hence, the two markers will subsequently be discussed without discriminating between them.

The most striking difference seen with the ecmAO:Rm aggregates is that lacZ staining increases in intensity starting at the base of the developing structure and extending gradually upward (Figs. 2B and 2G). This is in complete contrast to the development of normal cells, where the tip of the aggregate is the most strongly stained region at all times during its elongation (Figs. 2E and 2J). The aberrant staining becomes even more pronounced at later stages in slug formation, when wave-like distributions of staining cells appear, with maxima and minima at apparently random positions along the first finger (Figs. 2C and 2H). Later during slug migration most of the slugs show apparently uniform staining throughout their length (Figs. 2D and 2I), although the occasional slug similar to the large structure in Fig. 2C can be observed (data not shown).

In ecmAO:Rm slugs containing prestalk markers there are an excessively high number of stained cells and most of these must also have expressed a prespore-specific marker. The whole-mount staining patterns give the impression of a very high proportion of expressing cells at the slug stage (Figs. 2D and 2I) and this was confirmed by disaggregating migrating slugs and counting the proportions of stained cells. All of the cells are stained, using either marker (Figs. 3A and 3B), while in a normal slug only about 10% of cells express ecmA:lacZ and 10% express ecmO:lacZ (Early *et al.*, 1993, 1995). Thus, far from displaying a repression of prestalk cell differentiation, the ecmAO:Rm slugs seem to contain a great overabundance of ecmA:lacZ- and ecmO:lacZ-expressing cells.

The straightforward explanation for the large number of ecmA- and ecmO:lacZ-expressing cells is that they derive from the transdifferentiation of prespore cells. This was tested by analyzing slugs for the fraction of cells that stain with an antibody specific for vesicles (psv) that are present only in prespore cells (Fig. 3C). About 80% of cells stained with the anti-psv antiserum, the percentage expected for a normal slug (Hayashi and Takeuchi, 1976). Therefore, most of the cells in the ecmAO:Rm slug must at some time have expressed both a prespore and a prestalk marker. The conclusion that most cells show both prestalk and prespore characteristics was tested directly by double staining using a different marker of prespore differentiation.

PSPA, the product of the pspA gene, is a surface protein of prespore cells. In initial experiments, double immunohistochemical staining was performed using MUD-1, a monoclonal antibody directed against PSPA (Krefft *et al.*, 1983), and a rabbit polyclonal antibody directed against β-galactosidase. In the ecmO:lacZ control slugs prestalk and prespore cells were clearly discriminated (data not shown) but in the case of ecmAO:Rm/ecmO:lacZ cells it proved impossible to detect β-galactosidase immunohistochemically, because the level of lacZ gene expression was too low. Therefore, ecmAO:Rm/ecmO:lacZ cells were first stained enzymatically with lacZ and were then incubated with MUD-1 and secondarily stained with a fluorescent antibody. As expected, all cells (visualized under phase contrast in Fig. 4A and stained with X-gal in Fig. 4B) show lacZ expression and about 60–70% of these cells show detectable PSPA expression (Fig. 4C). This is slightly lower than with the anti-psv antiserum and this presumably indicates that MUD-1 staining is a marginally less sensitive detection procedure.

Cells within the prestalk regions of ecmAO:Rm slugs are rapidly replaced by cells from the posterior. The presence of cells that contain both β-galactosidase and PSPA (Fig. 4C) is a direct demonstration that there are cells within the population displaying both prespore and prestalk characteristics. The wave-like distribution of β-galactosidase-containing cells in the ecmAO:Rm/ecmO:lacZ and ecmAO:Rm/ecmA:lacZ first fingers, and the high proportion of apparent prestalk and prespore marker-coexpressing cells in the slug, suggests that there are cycles of transitory prestalk differentiation. Successive cohorts of prespore cells transdifferentiate and move to the anterior region. They then accumulate sufficient Rm protein to saturate the C subunit and revert to some, as yet undefined, state and fall backward. If true, then the forward flow of cells should be higher in ecmAO:Rm slugs and this notion was tested in two different grafting experiments.

Because of the evidence that the flow of cellular differentiations during normal slug migration is prespore into pstO then pstO into pstA (Abe *et al.,* 1994), we first used slugs wherein the pstO cells were fluorescently marked. A transformant strain containing the ecmAO:Rm construct was used to prepare slugs and the approximate front one-third parts of such slugs were grafted to the rear two-third parts of slugs prepared using a wild-type (i.e., non-Rm-containing) strain (Fig. 5a). The wild-type strain was marked by its expression of ecmO:GFP, a fusion gene in which the pstO-specific region of the ecmA promoter directs expression of GFP, the green fluorescent protein (Chalfie *et al.,* 1993). The control was to graft an unmarked, wild-type tip to such a fluorescently labeled rear.

In the control slugs there was very little forward movement of fluorescent cells, during a 2-hr period, while by 2 hr of migration the ecmAO:Rm slugs were uniformly fluorescent (Fig. 5b; N.B., because of its smaller size, the whole of the ecmAO:Rm slug is shown while, at this magnification, only the approximate front one-third of the AX-2 slug is visible). Thus, there is, as expected, a high rate of forward movement of cells into the prestalk region of ecmAO:Rm slugs.

To show that the cells which enter the front ultimately derive from the prespore population, a similar grafting experiment was performed using rear sections containing cells transformed with pspA:GFP. This construct contains the promoter of pspA, a prespore-specific gene, fused to GFP. To allow sufficient time for transdifferentiation, the slugs were analyzed 6 hr after grafting. Some forward movement is expected in the control, because β-galactosidase-containing cells are found in the prestalk regions of unmanipulated AX-2 slugs derived from pspA:lacZ-transformed cells (Harwood *et al.,* 1991; Detterbeck *et al.,* 1994). However, the difference from the ecmA:Rm, especially in the slug tips, is very clear; there are many fluorescent cells in the tip of the ecmA:Rm anterior portion, while there are only a very few fluorescent cells in the tip of the AX-2 anterior portion (Fig. 5c, N.B., in the slug with the ecmA:Rm tip most of the prespore region is out of the focal plane; see figure legend).

The level of an activity that binds to an essential regulatory element within the ecmA promoter is reduced in ecmAO:Rm cells. Having established that ecmAO:Rm cells are defective in prestalk cell differentiation, we determined whether the activity of a potential component of the DIF signaling pathway is altered in these cells. Within the region of the ecmO promoter that directs expression in pstO cells there is a 53-nucleotide subregion (the 53-mer) that will direct weak prestalk-specific expression, when it is multimerized and placed upstream of a heterologous cap site and TATA box (Kawata *et al.,* 1996). If the 53-mer is combined with a GT-rich sequence element, which is believed to act as a nonspecific amplifier of the level of gene expression, then the level of expression is greatly increased and it becomes possible to detect induction by DIF. The 53-mer contains an 11-nucleotide region that is perfectly homologous to a sequence within a cap-site proximal region of the ecmA promoter that is essential for expression in pstA cells. This 11-nucleotide region contains tandem repeats of the sequence TTGAA with an intervening A residue. Mutation of the two G residues inactivates the 53-mer. There is a developmentally regulated, nuclear localized DNA binding activity that binds to the 53-mer and competition assays show that it interacts with the TTGA repeats (Kawata *et al.,* 1996).

AX-2 cells and ecmAO:Rm cells were allowed to develop until they had just formed slugs and used to prepare nuclear extracts. Equal amounts of the protein preparations were then used in gel retardation with the 53-mer as a probe. As a test of specificity parallel binding reactions were performed with and without competitor. In extracts from AX-2 cells there was a retarded band that became reduced in intensity when unlabeled probe, or a probe containing mutations outside the 11-nucleotide region of homology, was used as competitor (Fig. 6). However, there was no competition with a version of the 53-mer wherein the two G residues that lie within the TTGA sequences are mutated. Extracts from ecmAO:Rm cells showed exactly the same responses with the different competitors but the strength of the uncompleted band was always four- to fivefold lower than that in AX-2 extracts (Fig. 6).

DISCUSSION

Interpretation of these results relies on the assumption that the Rm protein is a specific inhibitor of the C subunit of PKA and the evidence for this derives from the several previous studies using Rm. When expressed from various developmentally regulated promoters the Rm protein produces major developmental changes but control constructs containing Rc, a protein in which the domain that interacts with the C subunit is deleted, have no detectable effects (Harwood *et al.,* 1992a,b; Hopper *et al.,* 1993, 1995; Bonner and Williams, 1994; Hopper and Williams, 1994. This suggests very strongly that Rm functions via its inhibitory effect on the C subunit rather than by an effect on some other cellular component.

Analysis of the development of ecmAO:Rm cells is complicated by the fact that there is an inherent circularity in the experimental design; cells start to express the Rm protein only *after* they are induced to become prestalk cells. There is, however, a compensating advantage in this approach in that we can be sure that the effect of PKA is direct. It cannot be the result of a requirement for PKA at some earlier step in the developmental pathway, because the dominant inhibitor is not expressed until a cell actually becomes a prestalk cell.

This circularity presumably explains why, in the monolayer assay, there is a very low but detectable level of prestalk expression in cells where PKA is blocked. The expression that occurs must reflect the lag time before Rm accumulates to a sufficient level to inhibit prestalk gene expression. The situation during normal development is more complex than in the monolayer assay, because there

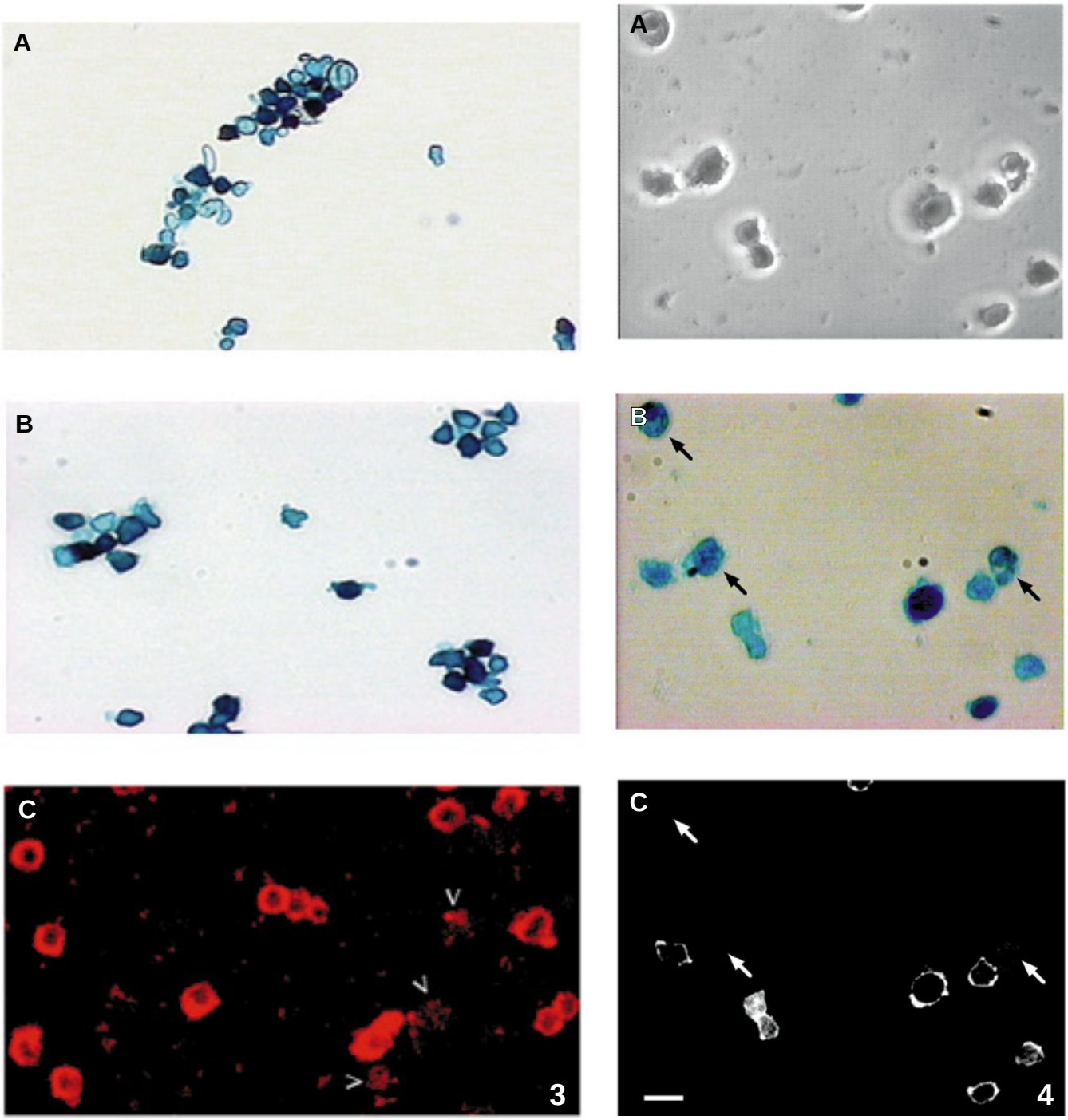


FIG. 3. Analysis of prestalk and prespore differentiation in ecmAO:Rm slugs. Cells doubly transformed with ecmAO:Rm and either ecmA:lacZ (A) or ecmO:lacZ (B) were used to prepare slugs and, after approximately 2 to 3 hr of migration, they were dissociated into single cells and stained overnight for β-galactosidase activity. Almost all cells on the slide show staining for both lacZ markers. There was no blue staining in control, untransformed cells (data not shown), so even the most weakly stained cells are deemed positive. The variation in staining intensity is most likely to be the result of different cells having expressed the ecmA gene for different lengths of time, because these are clonal populations. C shows cells dissociated from an ecmAO:Rm slug in this case stained for the presence of prespore vesicles. At this low power, the cells show a "doughnut" appearance that is caused by the absence of stain within the nucleus. The arrows indicate the positions of unstained cells that act as internal negative controls. A parallel slide where only the secondary antibody was used provided a further negative control and this displayed almost no fluorescence at this sensitivity level (data not shown). In a normal slug approximately 10% of cells express ecmA:lacZ, 10% express ecmO:lacZ, and 80% contain prespore vesicles (Early *et al.*, 1993, 1995; Hayashi and Takeuchi, 1976).

is the possibility of regulation, so we have combined several lines of evidence to show a requirement for PKA.

First, there is the altered pattern of differentiation of ecmAO:Rm transformants. Tip formation is greatly delayed and there is a major change in the expression patterns observed using markers of pstA and pstO cell differentiation. One interesting effect of the ecmAO:Rm is to cause a complete overlap in expression of the pstA- and the pstO-specific markers. This suggests that PKA may be a part of the signaling pathway that leads to the bifurcation in prestalk cell differentiation. There is also an effect on the level of gene expression. Relatively large numbers of transformant clones needed to be screened to identify usable ecmA:lacZ- and ecmO:lacZ-expressing clones and these still showed a relatively lower level of staining than control cells.

It is important to realize that the inhibition by ecmAO:Rm in the monolayer induction assay appears obvious and dramatic because equivalent staining times were used for the populations under test, while the ecmAO:Rm cells that were developed on filters were stained for approximately 10 times as long as the control populations. It is also necessary to stress that we are not selecting aberrant subpopulations of the ecmAO:Rm transformant cells when we select for expressing clones. In the course of these experiments identical results were obtained using four different clones of ecmAO:Rm/ecmO:lacZ transformants and four different clones of ecmAO:Rm/ecmO:lacZ transformants. Also, in subsequent experiments (N. Zhukovskaya, unpublished results), using other subfragments of the ecmA promoter coupled to lacZ, a total of 16 different ecmAO:Rm clones were isolated and all of them show the same behavior as the clones described here.

Ectopic expression is also observed using an ecmAO:lacZ reporter gene, i.e., one that contains the entire promoter, but it differs in one interesting respect; here there is always stronger expression in the front half of the slug (data not shown). This explains why the ectopic expression was overlooked in the study of Harwood *et al.* (1994), where shorter staining times were used and where less highly expressing clones may have been isolated. It suggests that there are regulatory elements in the intact promoter that are missing from the ecmA:lacZ and ecmO:lacZ constructs and we are currently mapping these.

Our previous study showed that there is a 5-fold lower level of *ecmA* expression, as observed by direct RNA analysis (Harwood *et al.*, 1994). A very crude estimate of the degree of underexpression can be made by combining the estimate of a 5-fold lower mRNA level in ecmAO:Rm slugs with the staining data (Fig. 3) showing that all cells express, or at one time did express, the gene. This calculation suggests that each cell expresses the gene at about a 25-fold lower level than control cells but it does not of course take into account the different lengths of time for which the gene may be being expressed in the control and ecmAO:Rm cells.

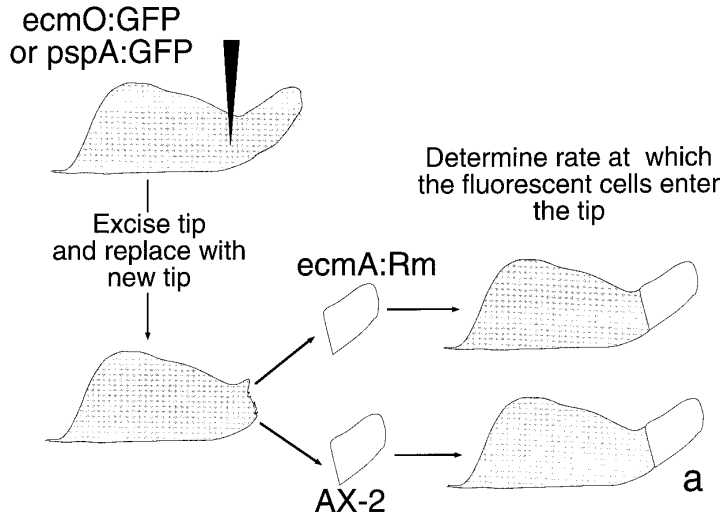
The delay in tip formation most probably reflects the change in the localization of prestalk cells which, rather than accumulating in the apex, predominantly populate the basal parts of the aggregate. We believe that these staining cells are cells that once expressed the ecmA gene and that may have had other characteristics of normal prestalk cells, but which reverted to an undifferentiated state once the Rm protein accumulated to a high enough level. The staining patterns themselves provide part of the evidence for this.

The staining patterns suggest that waves of transdifferentiation occur, synchronously enlisting groups of prespore cells to become prestalk cells, and that these then dedifferentiate, also as a group. There are often unstained tips (e.g., Fig. 2C, larger structure) that are presumably composed of undifferentiated cells left behind when a cohort of prestalk cells reverted and moved backward from the tip. Sometimes there are two bands of staining (e.g., Fig. 2H, lower structure) that are, we assume, two cohorts of prestalk cells at different stages in the transdifferentiation/reversion cycle. Later, after migratory slugs are formed most structures show uniform staining throughout (Figs. 2D and 2I).

Two additional, direct pieces of evidence suggest that this interpretation, of waves of transitory prestalk differentiation followed by reversion, is correct.

First, there are an abnormally high proportion of cells that are expressing, or which at one time in their history must have expressed, both prestalk and prespore markers. This is as expected if the prestalk population, effectively, disappears periodically. A fraction of the prespore cells will transdifferentiate to take their place. The indirect but very strong argument against the alternative interpretation that these cells first differentiated as prestalk cells and then became prespore cells derives from the design of the experiment. We used the ecmAO promoter fragment to express Rm and it does not direct expression in prespore cells. Therefore, during slug formation, the prespore cells should differentiate normally. Since we eventually find that all cells have at one time expressed the ecmA and ecmO markers it seems reasonable to assume that most of the comarked

FIG. 4. Analysis of the expression of the pspA gene in ecmAO:Rm cells by immunostaining. Cells doubly transformed with ecmO:lacZ and ecmAO:Rm were used to prepare slugs which were dissociated into single cells and affixed to glass slides. The amount of β-galactosidase protein proved to be too low to be detectable by immunofluorescence so orthodox double staining could not be performed. Therefore, cells were fixed in glutaraldehyde, stained overnight with X-gal, fixed again in methanol, and then sequentially stained using MUD-1 and a fluorescent second antibody. (A) A field of cells viewed by phase-contrast microscopy, (B) the same field observed by orthodox light microscopy, and (C) the same panel observed by fluorescence microscopy. The three blue-stained cells marked with an arrow in B do not show cell surface fluorescence in B and act as internal controls for the specificity of the staining. The seven cells showing both cell surface fluorescence and blue staining express (or at some time were expressing) the *lacZ* and the *pspA* genes.



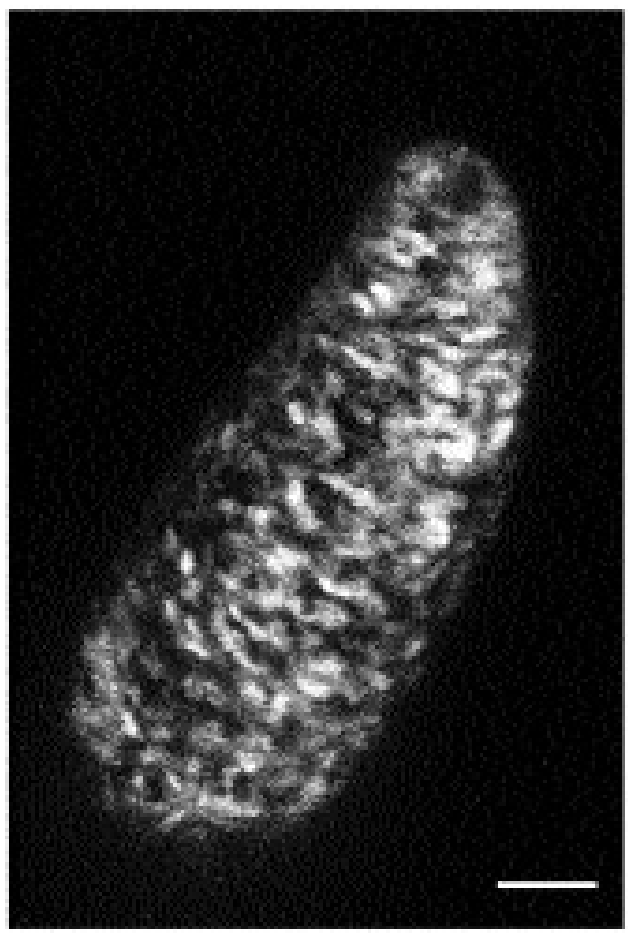


FIG. 5. Evidence for the transitory nature of prestalk cell differentiation in ecmAO:Rm slugs. (a) The principle of the two grafting experiments that are described in b and c. (b) Slugs were prepared from a transformant strain marked by its expression of ecmO:GFP, a fusion gene in which the pstO-specific region of the ecmA promoter directs expression of the green fluorescent protein (Chalfie *et al.*, 1994). The rear two-thirds of such slugs were grafted to the front one-third of untransformed AX-2 slugs (left-hand side) or of ecmAO:Rm slugs (right-hand side). After 2 hr the slugs were observed in a confocal microscope. The position of the AX-2 tip is shown by the white line. The ecmA:Rm slugs are on average much smaller than the AX-2 slugs and therefore, to keep a constant ratio of sizes, relatively small ecmO:GFP slugs were chosen as recipients for the grafting of ecmAO:Rm front portions. This explains why the whole grafted structure is shown for ecmA:Rm while only the approximate front one-third is show for AX-2.

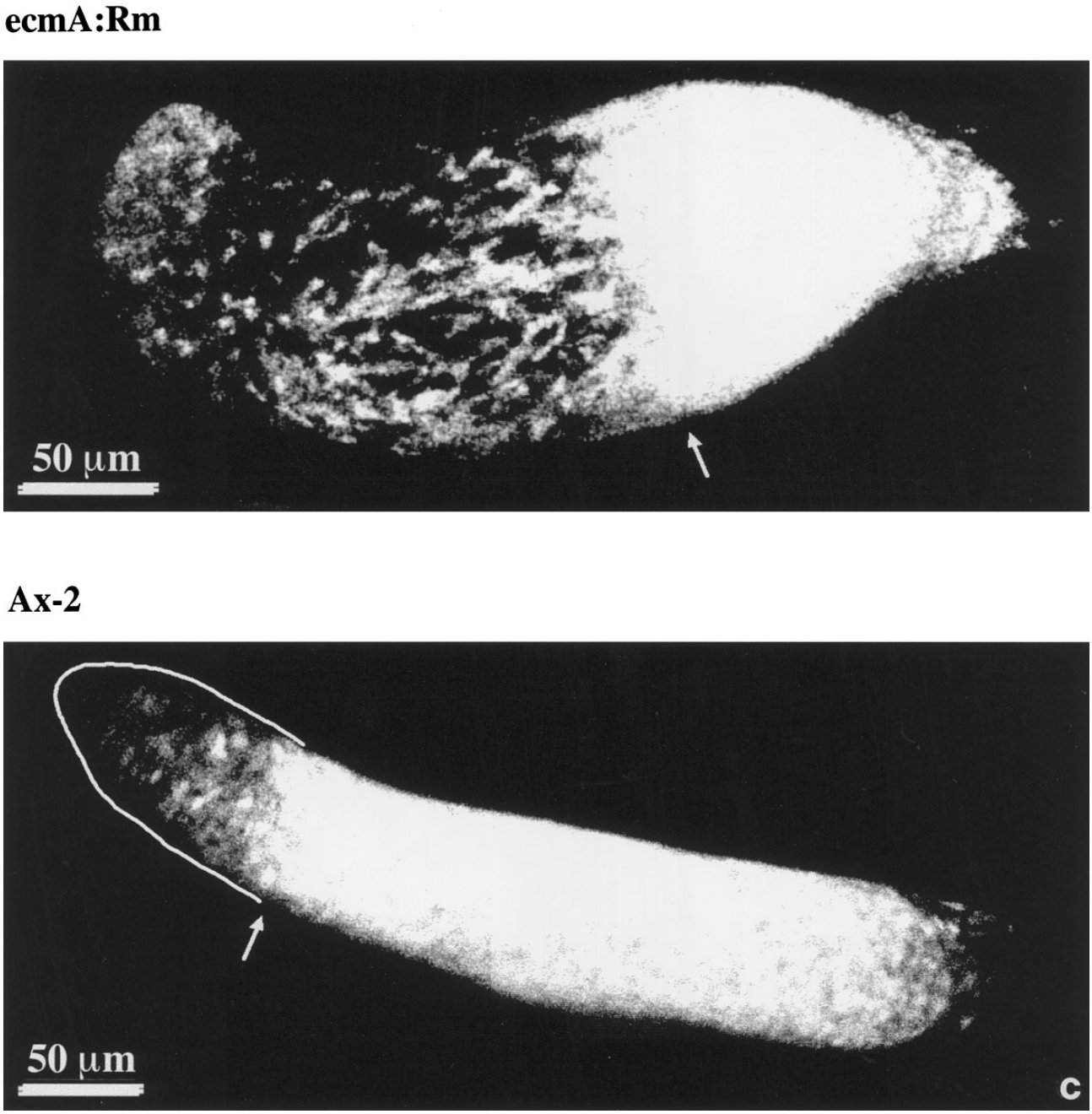


FIG. 5—*Continued* (c) Slugs were prepared from a transformant strain marked by its expression of pspA:GFP, a fusion gene in which the promoter of pspA, a prespore-specific gene, directs expression of the green fluorescent protein (Chalfie *et al.*, 1994). The rear two-thirds of such slugs were grafted to the front one-third of ecmAO:Rm slugs (top) or of untransformed AX-2 slugs (bottom). After 6 hr the slugs were observed in a confocal microscope. The position of the AX-2 tip is shown by the white line. The assumed position of the graft is shown by the white arrows, based on the demarcation between fluorescent and predominantly nonfluorescent cells. N.B., this is a plan view of a living slug that is arched (this is called ''hurdling'' and it is part of the movement cycle adopted by *Dictyostelium* slugs), such that most of the prespore region is out of the focal plane.

cells started off as prespore cells and transitorily became ecmA-expressing cells.

The second piece of experimental evidence supporting the notion of waves of transdifferentiation and forward movement comes from the two grafting experiments. Given that the flow of differentiation in the slug is prespore to

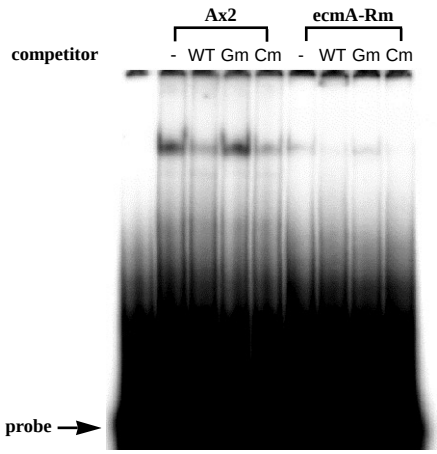


FIG. 6. Analysis of the activity of the TTGA binding protein in normal and ecmAO:Rm first fingers. A nuclear extract prepared from newly migrating AX-2 and ecmAO:Rm slugs was used in gel retardation with a 53-nucleotide probe (53-mer) that contains direct repeats of the TTGA sequence. Two micrograms of extract was used in each retardation assay. In those samples where competitor was included it comprised 100 ng of unlabeled 53-mer containing no mutations (lanes 2 and 7, WT), mutations in each of the two G residues in the TTGA direct repeats (lanes 3 and 8, Gm), and mutations in a block of C residues that have no known function (lanes 3 and 9, Cm). This serves as a second control for the competition and, as expected, it behaves like an unmutated 53-mer. Lane 1 contains no extract, lanes 2–5 contain AX-2 protein, and lanes 6–9 contain ecmA:Rm protein.

pstO, followed by pstO to pstA (Abe *et al.*, 1994), then loss of (or effective loss of) the tip should result in a forward flow of pstO/ALC and this is exactly what we observe (Fig. 5b). These pstO/ALC will be replaced by transdifferentiation of prespore cells and this also occurs at a much higher rate than in an AX-2 to AX-2 graft (Fig. 5c).

The grafting experiment may also help to explain some of the biological properties of the ecmAO:Rm slug. Tip dominance, the ability of the slug tip to control a cohort of prespore cells, determines slug size. EcmAO:Rm slugs are much smaller than normal and the grafting experiment suggests that the tip is indeed defective in this property. Their poor phototaxis is also, perhaps, explained by the transitory nature of the prestalk population within the tip and their incomplete state of differentiation.

Why is there a requirement for PKA? On the prespore pathway there is evidence to suggest that PKA acts to control gene expression by acting on, or upstream of, GBF (Hopper *et al.*, 1995), a transcription factor that binds to GT-rich sequence elements present in many developmentally regulated genes (Schnitzler *et al.*, 1994). There are several potential G boxes in the ecmA gene (Early *et al.*, 1993) and the requirement for PKA could, therefore, reflect a requirement by GBF for PKA activation.

Even if true, this is likely to be only a partial explanation of the effects of PKA because GBF does not endow cell-type specificity of gene expression; G boxes are present in both cell type-specific and non-cell type-specific promoters. The fact that inactivation of PKA renders pstA- and pstO-specific constructs equivalent argues for a cell type-specific effect of the kinase. The tandemly repeated TTGA elements within the ecmA promoter interact with nearby GT-rich sequence elements to direct DIF-inducible, prestalk-specific gene expression (Kawata *et al.*, 1996). The fact that the activity which binds to these elements is reduced in first fingers derived from ecmAO:Rm slugs suggests that the DIF signal transduction pathway involves a PKA-dependent step.

One final word of caution is required. We cannot be entirely certain that PKA is directly involved in either the GBF pathway of the DIF induction pathway. The inhibitory activity of Rm could reflect an indirect effect on another signaling pathway. For example, PKA is required for the activation of adenylate cyclase in response to extracellular cAMP signals (Harwood *et al.*, 1992a) and disruption of cAMP signaling by the prestalk cells could lead to their own dedifferentiation. Proof that PKA is directly involved will require a demonstration that it phosphorylates a known component of one or another of the two pathways.

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